Mitochondrial Effect of Orally Administered Dibutyl Phthalate in Rats

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INTRODUCTION

Phthalate esters (PAEs) are used as plasticizers for polyvinyl chloride and widely distributed in our environment. Recently health hazards caused by PAEs have been reported¹⁻³⁾.

Among the toxic effects of PAEs, mitochondrial effects have been studied by the following researchers. Lake et al.⁴⁻⁶⁾, Srivastava et al.⁷⁾, and Mann et al.⁸⁾ observed inhibited succinate dehydrogenase activity in liver homogenate from di-2-ethylhexyl phthalate (DEHP)-treated rats. Ohyama⁹⁾ showed *in vitro* that PAEs inhibited mitochondrial respiration, with di-n-butyl phthalate (DBP) showing the strongest effect, and he concluded that PAEs were inhibitors of electron and energy transfer systems but not uncouplers. Takahashi¹⁰⁾ also found inhibitory effects of PAEs on respiration *in vitro* and ascertained that PAEs acted mainly as energy-transfer inhibitors. Then, Inouye et al.¹¹⁾ reported that PAEs acted *in vitro* as uncouplers rather than electron-transfer inhibitors.

Thus, these reports did not always coincide in terms of the site of action of PAEs on the mitochondrial respiration. In order to determine the inhibitory site of PAEs in mitochondrial respiration, we studied the effects of DBP on respiratory activity and dehydrogenases activities in rat liver mitochondria *in vivo* and *in vitro*.

MATERIALS AND METHODS

In vivo: Five male rats of the Wistar strain, about 320 g in weight were fed a powder diet containing 5% DBP *ad libitum*. Pair feeding was also carried out in studying the dehydrogenase activity as described below. From the 35th to 45th experimental days, the rats were killed by decapitation and blood letting, and the liver, kidney, spleen and testicles were removed and weighed. Liver mitochondria were isolated by the Hogeboom method¹²⁾, and the protein content was determined by measuring the content of cytochrome *a* + *a*₃ using a Hitachi, Model 556, two-wavelength, double-beam spectrophotometer^{13,14)}.

Respiratory activity was determined by the ordinal method¹⁵⁾ using a Yellow Spring, Model YSI53, oxymeter. The Medium A (4.5 ml) consisted of 5 mM MgCl₂, 2 mM EDTA, 15 mM KCl, 50 mM sucrose, 25 mM Tris-HCl buffer (pH 7.4), and 20 mM KH₂PO₄-K₂HPO₄. As a substrate, succinate (with 2 μg of rotenone), pyruvate (with 5 mM L-malate) or glutamate (with 5 mM L-malate), 10 mM each was used. ADP and 2, 4-dinitrophenol (DNP) were used at concentrations of 250 μM and 50 μM, respectively.

Dehydrogenase activity for each of these substrates was determined by the amount of reduced potassium ferricyanide¹⁶⁾ as measured with wavelength pair of 450 nm minus 500 nm in a Hitachi, Model 556, two-wavelength, double beam spectrophotometer. The concentration of mitochondria used was 1 mg protein per ml medium. The medium (3.0 ml) used was the same as the Medium A except that 1 mM KCN, 20 mM potassium ferricyanide, and 40 μM DNP were also added. The substrates used were the same as those in the respiration study.

In vitro: Using mitochondria from intact rat liver, the effects of DBP on respiratory and dehydrogenase activities were observed *in vitro*. The effect on ATP production was also observed using

submitochondrial particles prepared by the Hansen and Smith method¹⁷⁾, as described previously¹⁸⁾. The submitochondrial particles used were at a concentration of 1 mg protein per ml medium. The medium (1.5 ml) used was the same as the Medium A except that 10 mM glucose, 0.1 mg hexokinase, and 20 mM Pi containing about 10^6 cpm ^{32}Pi were also added.

RESULTS

General findings

Food consumption by DBP-treated rats was less than that of the control during the first week, then it recovered to the control level. Body weight showed a depressed gain during the first week, followed by a plateau, measuring 65% of the control value on the 35th day (Fig. 1). Organ weights are shown in Table 1. The liver and spleen of treated rats presented no significant change in absolute weight, but a significant increase in relative weight. On the other hand, the testicle presented marked atrophy, showing 40% of the control value in absolute weight and 60% of the control value in relative weight.

Respiratory activity in liver mitochondria

Table 2 shows the respiratory activities in DBP-treated rat liver mitochondria. As shown in this Table, state 4 respiration was not significantly inhibited. The state 3 respiration was significantly inhibited in the presence of succinate or pyruvate. The P/O ratio, however, did not differ from that of the control. DNP-induced oxygen uptake was almost the same percent of the control value as that seen in state 3.

The results obtained *in vitro* are shown in Table 3. At concentrations of 50 to 100 μM DBP, inhibition of state 3 respiration was the same as that seen *in vivo*. State 4 respiration did not change at a concentration lower than 100 μM DBP, but was weakly stimulated at a higher concentration. Respiratory control was completely lost at higher than 150 μM .

Fig. 1 Growth curves for normal and DBP-treated rats. Conditions were as described under the Materials and Methods. Vertical lines indicate S.E.

Table 1 Organ weight of DBP-treated rats. DBP was administered as described under the Materials and Methods.

Organ	Control	DBP-treated	% of control
Absolute weight (g)			
Liver	16.4 $\pm$ 2.1	17.9 $\pm$ 1.7	109.1
Kidney	2.90 $\pm$ 0.27	2.51 $\pm$ 0.13	86.6
Spleen	0.72 $\pm$ 0.15	0.83 $\pm$ 0.19 ^b	115.2
Testicle	3.49 $\pm$ 0.26	1.42 $\pm$ 0.44 ^a	40.7
Relative weight (g/100 g of body weight)			
Liver	3.9 $\pm$ 0.4	6.8 $\pm$ 0.8 ^a	174.3
Kidney	0.70 $\pm$ 0.04	0.95 $\pm$ 0.07 ^a	135.7
Spleen	0.18 $\pm$ 0.03	0.32 $\pm$ 0.09 ^a	177.8
Testicle	0.84 $\pm$ 0.08	0.54 $\pm$ 0.18 ^a	64.3

Each value represents the mean $\pm$ SE of five animals. a, b: Significantly different from control (a: $p < 0.01$, b: $p < 0.05$).

Table 2 Respiratory activity in liver mitochondria of DBP-treated rats. Conditions were as described under the Materials and Methods. P : O ratios and respiratory control index (RCI=State 3/State 4) were calculated from the traces of oxygen uptake recorded according to the method of Hagihara²⁶.

	O ₂ uptake ^a		RCI	P : O	O ₂ uptake ^a
	State 4	State 3			DNP-induced
Succinate as a substrate					
Control	17.1±3.7	74.6±12.6	4.4±0.7	1.8±0.2	89.5±10.4
DBP-treated	15.6±6.0	47.7±12.3 ^c	3.1±0.8 ^c	1.8±0.1	55.3±10.3 ^b
% of control	91.2	63.9	70.5	100.0	61.8
Pyruvate as a substrate					
Control	3.4±0.6	16.2±2.8	4.8±0.1	2.7±0.2	17.3±2.8
DBP-treated	2.8±1.1	8.8±2.0 ^b	3.1±0.1 ^b	2.7±0.2	9.1 ±1.9 ^b
% of control	83.9	54.3	64.6	100.0	52.6
Glutamate as a substrate					
Control	10.1±5.7	47.5±12.6	4.7±0.6	2.8±0.2	51.3±10.9
DBP-treated	11.3±6.4	42.6±12.6	3.8±0.3	2.7±0.2	42.6±9.4
% of control	111.9	89.7	80.9	96.4	83.0

Each value represents the mean ±SE of five animals. a : Expressed as nanomole O₂ per minute per milligram protein. b, c : Significantly different from control (b : $p < 0.01$, c : $p < 0.05$).

Table 3 Effect of DBP *in vitro* on the respiration of liver mitochondria of normal rats. Succinate was used as a respiratory substrate. The other conditions were as for Table 2.

DBP (μM)	O ₂ uptake ^a		RCI	P : O	O ₂ uptake ^a
	State 4	State 3			DNP-induced
0	15.2±0.6	73.0±10.6	4.8±0.5	1.9±0.1	71.0±11.6
50	16.4±0.0	63.3± 0.1	3.9±0.6	1.9±0.1	64.8± 6.7
100	26.0±1.0	51.2± 1.0	2.0±0.0	1.2±0.2	49.5± 2.3
150	36.7±2.1	36.7± 2.1	1.0±0.0	—	36.7± 2.1
200	31.8±4.4	31.8± 4.4	1.0±0.0	—	31.8± 4.4
300	20.4±0.9	20.4± 0.9	1.0±0.0	—	20.4± 0.9
400	16.2±3.5	16.2± 3.5	1.0±0.0	—	16.2± 0.9

Each value represents the mean ±SD. a : Expressed as nanomole O₂ per minute per milligram protein.

Dehydrogenase activity in liver mitochondria

Dehydrogenases activities in DBP-treated rat liver mitochondria were shown in Table 4. Glutamate dehydrogenase activity was decreased by 73% of the control value but this was not significant. Succinate and pyruvate dehydrogenase activities were significantly decreased by 59% and 38% of the control values, respectively. These inhibitory effects were also observed in pair feeding rats, though they were somewhat weak compared with rats fed *ad libitum* (Table 4). The inhibitory potency for dehydrogenase activity was very close to that for respiratory activity shown in Table 2. Thus, these data clearly show that the respiratory inhibition by DBP administration was mainly caused by the inhibitory effect on dehydrogenase activity rather than on the other respiratory pathways.

The results obtained *in vitro* are shown in Table 5. Succinate dehydrogenase activity was more greatly inhibited as DBP concentration increased, and 100–200 μM DBP provided inhibition similar to that seen *in vivo*. ATP synthesis in submitochondrial particles (+succinate), which are inside-out relative to the membranes of mitochondria¹⁹, was also reduced by 41% of the control when 400 μM DBP was

added (Table 6). The degree of reduction was very close to that (35.1%) of dehydrogenase activity at the same DBP concentration, as shown in Table 5.

DISCUSSION

In the present study, DBP-treated rats revealed depressed growth, liver enlargement (increased

Table 4 Dehydrogenase activity in liver mitochondria of DBP-treated rats. Conditions were as described under the Materials and Methods.

	Activity of dehydrogenase ^a		
	Succinate	Pyruvate	Glutamate
Ad lib.			
Control	0.41±0.02	0.08±0.01	0.15±0.04
DBP-treated	0.24±0.02 ^b	0.03±0.00 ^b	0.11±0.02
% of control	58.5	37.5	73.3
Pair feeding			
Control	0.42±0.04	0.12±0.00	0.17±0.01
DBP-treated	0.28±0.06 ^b	0.07±0.01 ^b	0.14±0.02
% of control	66.7	58.3	82.4

Each value represents the mean±SE of five animals. a: Expressed as micromole reduced potassium ferricyanide per minute per milligram protein. b: Significantly different from control ($p<0.01$).

Table 5 Effect of DBP *in vitro* on the succinate dehydrogenase activity in liver mitochondria of normal rats. Conditions were as described under the Materials and Methods.

DBP(μM)	Activity ^a	%
0	0.77±0.04	100
100	0.54±0.03	70.1
200	0.44±0.03	57.1
400	0.27±0.02	35.1
1000	0.15±0.02	19.5

Each value represents the mean ±SD. a: Expressed as micromole reduced potassium ferricyanide per minute per milligram protein.

Table 6 Effect of DBP on ATP formation in sub-mitochondrial particles of normal rat liver. Conditions were as described under the Materials and Methods.

DBP(μM)	ATP formation ^a	%
0	43.5±2.1	100
400	18.0±1.4	41

Each value represents the mean ±SD. a: Expressed as nanomole ATP per minute per milligram protein.

relative weight) and testicular atrophy, effects common to previous reports^{4,5,20-23}. These animals also evidenced depressed respiration in liver mitochondria when succinate or pyruvate was used as a substrate. State 4 respiration was not, however, altered significantly, suggesting the absence of any effect on the coupling between respiration and phosphorylation at the given dose. The main inhibitory effects were seen in state 3 and DNP-induced respiration. However, the P/O ratios of DBP-treated and control mitochondria were almost the same. Moreover, the inhibitory effect on respiration was the same as that of succinate and pyruvate dehydrogenases activities.

Therefore, these findings clearly show that the respiratory inhibition by DBP administration was mainly caused by the inhibitory effect on succinate and pyruvate dehydrogenases rather than on the other respiratory pathways or the energy transduction system in mitochondria.

ATP synthesis in submitochondrial particles (+succinate), which are inside-out relative to the membranes of mitochondria¹⁹, was decreased by 41% of the control value at a concentration of 400 μM DBP, which was very close to the inhibition (35.1%) of succinate dehydrogenase activity in mitochondria at the same concentration of DBP. These results clearly show that DBP can be easily taken up into the matrix of mitochondria and that it acts directly on succinate dehydrogenase without altering the transport system for succinate.

Potent effects of DBP on liver mitochondrial function observed in the treated rats of this study were reproducible *in vitro*. This finding suggests that intact DBP, when elevated in the liver, contributes considerably to the development of adverse effects at least on the liver, especially since the existence of intact DBP in the liver a short time after an oral dose of DBP, though presumably in small amounts, has been suggested by analysis of metabolites in urine²⁴ and in the bile²⁵.

Since no definite information is available on the extent to which DBP plasticizer migrates from plastic materials into food or blood, it should be noticed that the significance of the present findings

in relation to human exposure remains to be determined.

SUMMARY

Rats fed a diet containing 5 % dibutyl phthalate (DBP) for 35 to 45 days revealed growth depression, liver enlargement and testicular atrophy. These effects were accompanied by inhibited respiration in liver mitochondria. This inhibition was caused by the decrease of succinate and pyruvate dehydrogenases activities rather than by the inhibition in the other respiratory redox components, in the coupling process or in the energy-transfer process. Such inhibitory effect was also observed *in vitro*, where nearly the same inhibitory potency as that found *in vivo*, occurred at a concentration less than 100 μ M DBP for the respiration and 150 μ M DBP for the dehydrogenase activities. The present study also showed that DBP can be easily taken up into the matrix of mitochondria and that it directly affects succinate dehydrogenase without altering the transport system for succinate.

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フタル酸ジブチル経口投与のラット肝ミトコンドリアに及ぼす影響

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ラットに5%フタル酸ジブチル(DBP)含有粉末飼料を35~45日間与えた結果、成長抑制、肝肥大、精巣萎縮が認められた。又、同時に肝ミトコンドリアの呼吸抑制が認められた。この呼吸抑制はコハク酸およびピルビン酸脱水素酵素の活性低下によるものであった。

このようなDBPのミトコンドリアに及ぼす影響は *in vitro* でも確認された。即ち *in vivo* で観察された呼吸活性の低下と同程度の低下はDBP 100 μ M以下の濃度で認められ、又同様にコハク酸脱水素酵素活性の低下は150 μ Mで認められた。

又、亜ミトコンドリア粒子を用いた実験結果から、DBPは容易にミトコンドリア内に入り、コハク酸の膜透過には変化を与えず、コハク酸脱水素酵素活性に直接作用することが示された。

Key words: Dibutyl phthalate, Toxicity, Rat liver, Mitochondrial respiration, Dehydrogenase activity

フタル酸ジブチル, 毒性, ラット肝, ミトコンドリア呼吸, 脱水素酵素活性

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